The Patent Office

09 JUNE 1999

INVESTOR IN PEOPLE

2171961

GB99/01824

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed R. Mahoney

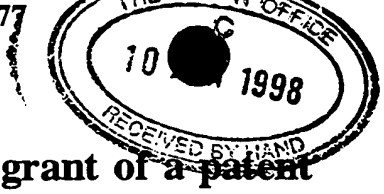
Dated -2 JUL 1999

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patents Form 1/77

Patents Act 1977
(Rule 16)



The Patent Office

1/77

10JUN98 E367248-2 002823
P01/7700 25.00 - 9812523.0

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference	KMN/FP5691530
2. Patent application number *(The Patent Office will fill in this part)*	**9812523.0** 10 JUN 1998
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI SPA VIA PONTINA KM. 30.600 00040 POMEZIA (ROMA) ITALY
Patents ADP number *(if you know it)*	7439219004
If the applicant is a corporate body, give the country/state of its incorporation	ITALY
4. Title of the invention	PEPTIDE INHIBITORS OF HEPATITIS C VIRUS NS3 PROTEASE
5. Name of your agent *(if you have one)*	MEWBURN ELLIS
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	YORK HOUSE 23 KINGSWAY LONDON WC2B 6HP
Patents ADP number *(if you know it)*	109006

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month /year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *See note (d))*	YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	0
Description	41
Claim(s)	0
Abstract	0
Drawing(s)	0

10. If you are also filing any of the following, state

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	0
Request for preliminary examination and search *(Patents Form 9/77)*	0
Request for substantive examination *(Patents Form 10/77)*	0
Any other documents *(Please specify)*	0

11. I/We request the grant of a patent on the basis of this application.

Signature: Kathryn Nicholls

Date: 9 June 1998

12. Name and daytime telephone number of person to contact in the United Kingdom: KATHRYN M. NICHOLLS 0117 926 6411

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed it it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

PEPTIDE INHIBITORS OF HEPATITIS C VIRUS NS3 PROTEASE

This invention relates to compounds which can act as inhibitors of the hepatitis C virus (HCV) NS3 protease, to uses of such compounds and to their preparation.

The hepatitis C virus (HCV) is the major causative agent of parenterally-transmitted and sporadic non-A, non-B hepatitis (NANB-H). Some 1% of the human population of the planet is believed to be affected. Infection by the virus can result in chronic hepatitis and cirrhosis of the liver, and may lead to hepatocellular carcinoma. Currently no vaccine nor established therapy exists, although partial success has been achieved in a minority of cases by treatment with recombinant interferon-á, either alone or in combination with ribavirin. There is therefore a pressing need for new and broadly-effective therapeutics.

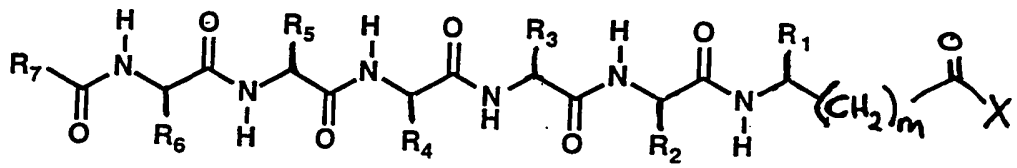
Several virally-encoded enzymes are putative targets for therapeutic intervention, including a metalloprotease (NS2-3), a serine protease (NS3), a helicase (NS3), and an RNA-dependent RNA polymerase (NS5B). The NS3 protease is located in the N-terminal domain of the NS3 protein, and is considered a prime drug target since it is responsible for an intramolecular cleavage at the NS3/4A site and for downstream intermolecular processing at the NS4A/4B, NS4B/5A and NS5A/5B junctions.

Previous research has identified classes of peptides, in

particular hexapeptides, showing degrees of activity in inhibiting the NS3 protease. The aim of the present invention is to provide further compounds which exhibit similar, and if possible improved, activity.

The present inventors have investigated the replacement of cysteine by 2,2-difluoro-1-aminobutyric acid or 2,2,2-trifluoro-1-aminobutyric acid at the P1 position of certain peptidic product inhibitors and substrates of HCV NS3 serine protease. These studies have shown that fluorocarbon groups, in particular the $-CF_2H$ group may mimic the cysteine thiol group, which is believed to be involved in substrate and inhibitor binding to the S1 specificity pocket of the NS3 protease. In general terms, therefore, the present invention relates to compounds containing fluorocarbon groups, especially $-CF_2H$ and $-CF_3$, for use as inhibitors of HCV NS3 protease. Examples of such compounds include peptides or peptide analogs, such as hexapeptides, in which a fluorocarbon group, such as $-CF_2H$, is present as a sidechain, for instance at the C-terminus or P1 position of the peptide.

In particular, according , to a first aspect of the present invention there is provided a compound of formula 1:



FORMULA 1

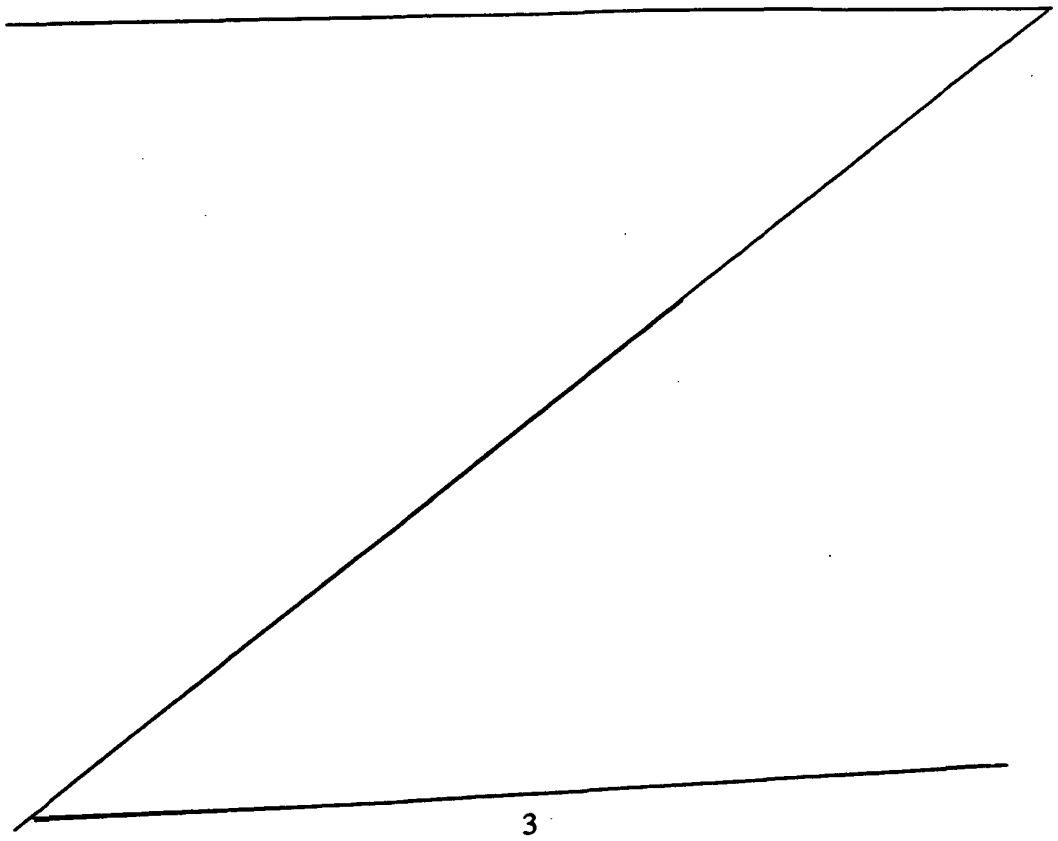
the substituents of which are defined below. Optionally, the compound may be in the form of a pharmaceutically acceptable salt. Alternatively it may be in the form of a pharmaceutically acceptable ester.

Formula 1 defines a class of hexapeptides and hexapeptide analogs, examples of which have been found to act as inhibitors of the HCV NS3 protease. The compounds are characterised in particular by the nature of the group R_1, ie, by the amino acid or amino acid analog found at the C- terminus of the hexapeptide.

In formula 1,

m is 0 or 1

R_1 is a group containing 1 to 6 carbon atoms and which



includes a fluorocarbon group such as $-CF_3$ or $-CF_2H$;

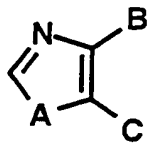
R_2 and R_3, which may be the same or different, are groups containing hydrocarbon fragments (which may include aromatic groups), and/or acidic functionality;

R_4 is a group containing a lipophilic fragment;

R_5 and R_6 may be the same or different and are groups containing an acidic functionality (the acid group may be present in the protonated or unprotonated (ie, carboxylate) form);

R_7-CON represents an amide, urethane or urea, in which R_7 is a group containing 1 to 8 carbon atoms; and

X is OH, CF_3, H, COOH, $CONR_9\ R_{10}$, $CF_2CONR_9\ R_{10}$, or a heterocyclic group, which can be of formula 2:



Formula 2

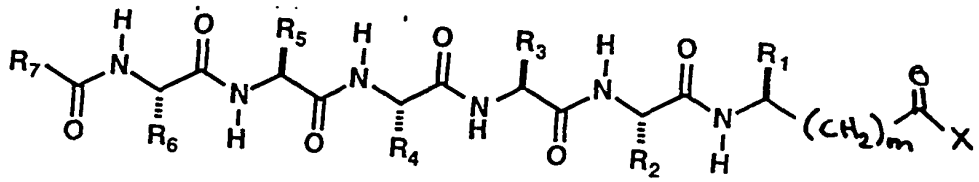
in which A = sulphur, oxygen or NR_8,
R_8, R_9 and R_{10} are, independently, hydrogen or any suitable aliphatic or aromatic, optionally substituted, groups, such as in particular alkyl or aralkyl groups, typically having between 1 and 8 carbon atoms, and
B and C are each independently either H or R_{11}, where R_{11} is an alkyl or aralkyl group, typically having between 1

and 8 carbon atoms, or can together form part of a ring, preferably an aromatic ring such as a phenyl ring.

The term "acidic functionality" as used herein encompasses not only carboxylic acid groups but also acid mimetics such as tetrazoles and acylsulphonamides.

The groups R_1-R_6 may be the side chains of naturally-occurring amino acids, or other, preferably analogous, groups having the requisite functionality. Such groups may contain, in addition to carbon and hydrogen, heteroatoms such as nitrogen, oxygen, sulphur and phosphorous; they may be saturated or unsaturated, branched or unbranched, substituted or unsubstituted. Their substituents can include halogens and/or other inorganic elements. Groups of this type preferably contain between 1 and 20 carbon atoms, more preferably between 1 and 13 carbon atoms, particularly preferably between 1 and 8 carbon atoms.

Each amino acid, or amino acid analog may have D- or L-stereochemistry, but L-stereochemistry is preferred. Preferably, all five N-terminal amino acids or analogs are L- isomers. The stereochemistry at the R_1 position allows either the D or the L isomer, of which the L isomer is usually preferred. It is particularly preferred that all six amino acids, or analogs are L-isomers. Thus, a preferred stereoisomer of formula 1 is as shown overleaf:



The compound may be a mixture of stereoisomers, especially of diastereomers having different stereochemistry at the position of R_1 substitution only.

Preferred substituents for formula 1 include:

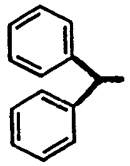
m=0

for R_1: $-CH_2CF_3$ or, more preferably, $-CH_2CF_2H$;

for R_2: the side chain of an acidic amino acid, especially $-CH_2CH_2CO_2H$ (the side chain of glutamic acid), or the side chain of an amino acid having a non-polar side chain such as alanine, isoleucine, valine or, in particular, $-CH_2CH(CH_3)_2$ (from leucine), or, more preferably, the side chain of β- cyclohexylalanine:

for R_3: the side chain of an acidic amino acid, for instance $-CH_2CH_2CO_2H$ (glutamic acid), or a non-polar amino acid side chain such as $-CH(CH_3)_2$ (from valine), $-CH_2CH(CH_3)_2$ from leucine) or $-CH(CH_3)(CH_2CH_3)$ (from isoleucine), preferably glutamic acid;

for R_4: the side chain of an amino acid having a hydrophobic R group, for instance - CH_2CH_2SMe (from methionine), or the side chains of leucine, isoleucine, or more preferably diphenylalanine:



for R_5 and R_6: $-CH_2CH_2CO_2H$ (from glutamic acid) or - CH_2CO_2H (from aspartic acid);

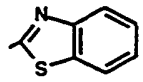
for R_7: - CH_3; and

for R_8: -H;

for R_9: -H;

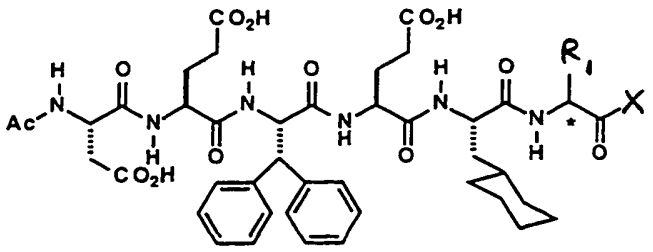
for R_{10}: benzyl, phenethyl; and

for X: $-CO_2H$, - $CONHCH_2Ph$,

 ,  ,

or more preferably H, or -OH.

A most preferred group of compounds according to the invention can be represented by the general formula 3:

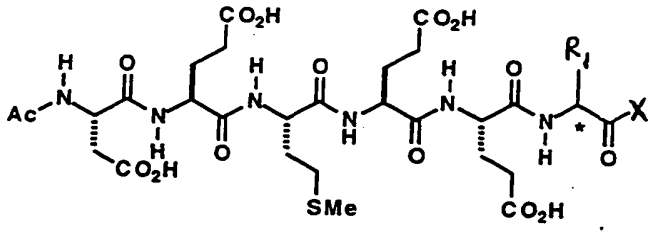


FORMULA 3

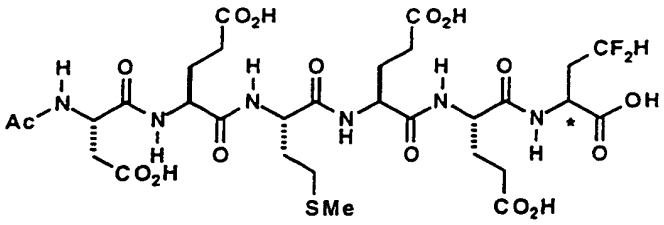
in which R_1 and X are as defined above.

Another preferred group of compounds is represented by the general formula 3' in which, again, R_1 and X are as defined above

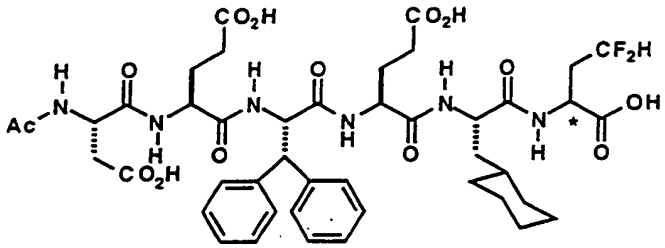
FORMULA 3'



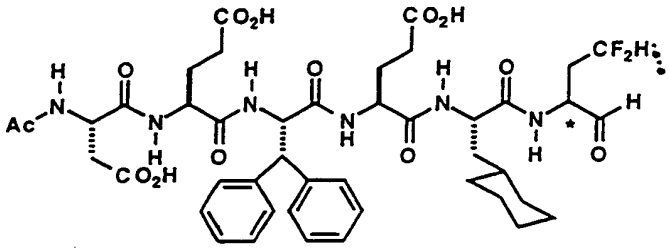
Specific compounds within the scope of the present invention include:


Ac
SMe
OH
CF2H

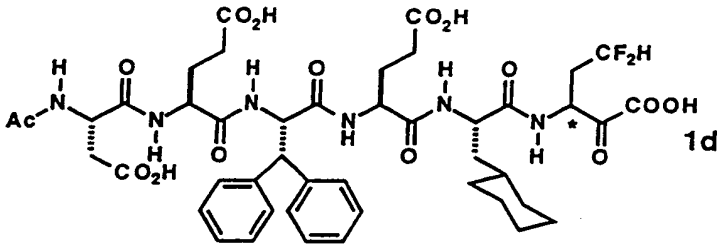
1a


Ac
OH
CF2H

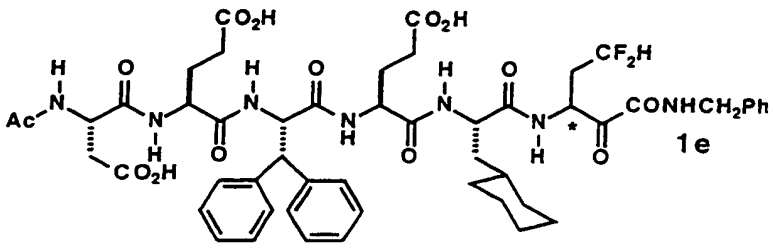
1b


Ac
CF2H

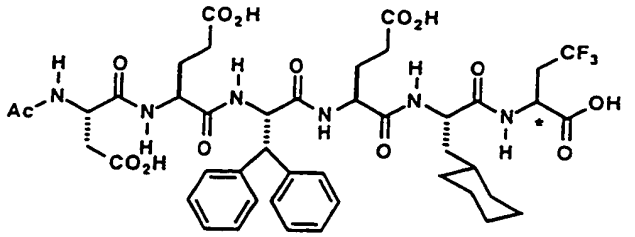
1c


Ac
COOH
CF2H

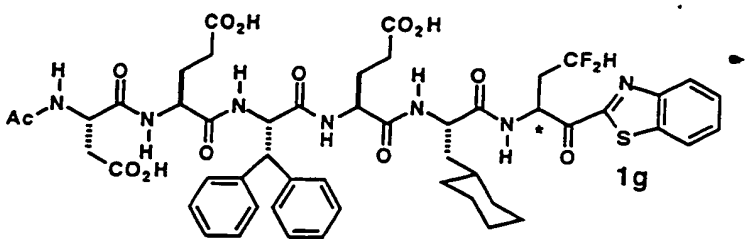
1d


Ac
CONHCH2Ph
CF2H

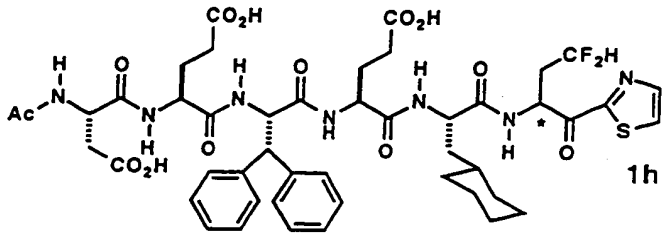
1e



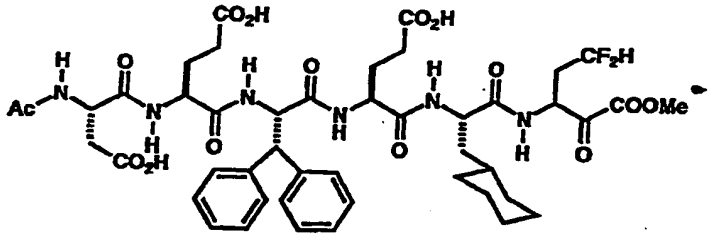
1f



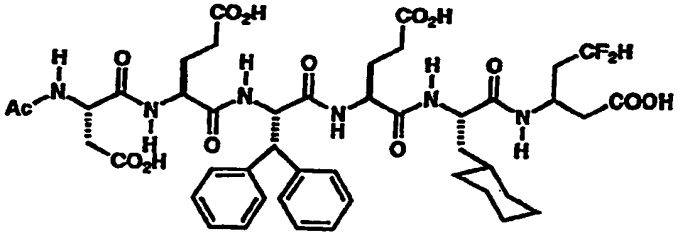
1g



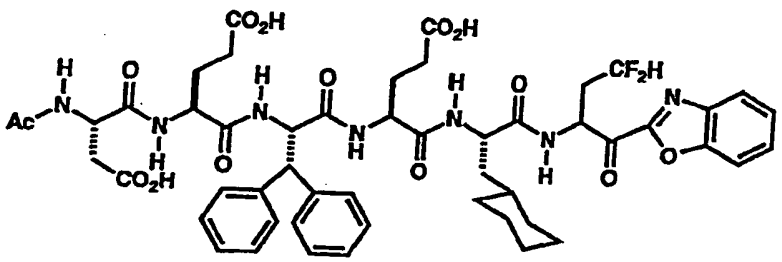
1h



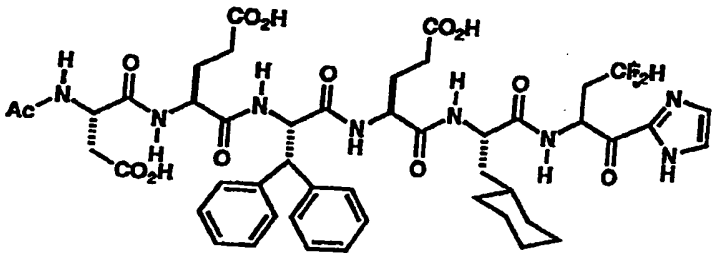
1j



1k



1l



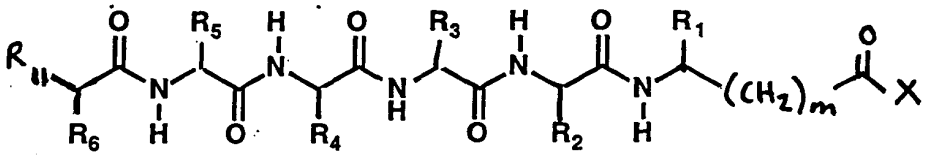
1m

These compounds, and analogous compounds within the scope of the invention, have been found to inhibit the action of the HCV NS3 protease at concentrations lower than 10 μM. They can therefore be expected to be of use in the treatment and prevention of hepatitis C and other related conditions.

The first aspect of the invention also provides a derivative of a compound of formula 1. "Derivative" includes a compound or composition in which the compound of formula 1 is bound to a coupling partner such as a label, a supporting substrate, a carrier or an effector molecule.

In particular, derivatives include "prodrug" forms of the compounds of Formula 1 which may be converted in vivo into the compound of Formula 1. Examples of such derivatives include those in which one or more carboxylic acid groups of the compound of Formula 1 are esterified or otherwise derivatised into groups convertible in vivo into carboxylic acid or carboxylate groups. For instance carboxylic acid groups may be esterified with C_1-C_{18} alcohols, preferably C_1-C_8 alcohols. Another possibility is that the derivative may be a C-terminal extended variant of the compound of Formula 1, convertible in vivo into a compound of Formula 1.

Another derivative within the scope of the present invention is modified at the N-terminus of the molecule and has the formula shown below as formula 1'



FORMULA 1'

where R_1-R_6, m and X are as specified above, and the same qualification concerning stereochemistry apply. However, the N-terminal amino acid, providing the group R_6, has been replaced by a simple carboxylic acid, with loss of the acylamino moiety. In formula 1', R_{11} is an optionally substituted lower alkyl group having from 1 to 6 carbon

atoms or, more preferably, is hydrogen. Preferred N-terminal carboxylic acids are glutaric or succinic acid.

According to a second aspect the present invention provides a compound or derivative according to the first aspect, for use in any therapeutic method, preferably for use in inhibiting the HCV NS3 protease, and/or for use in treating or preventing hepatitis C or a related condition. By "related condition" is meant a condition which is or can be caused, directly or indirectly, by the hepatitis C virus, or with which the HCV is in any way associated.

According to a third aspect the present invention provides the use of a compound or derivative according to the first aspect in the manufacture of a medicament for the treatment or prevention of hepatitis C or a related condition.

A fourth aspect of the invention provides a pharmaceutical composition which includes one or more compounds or derivatives according to the first aspect.

The composition may also include pharmaceutically acceptable adjuvants such as carriers, buffers, stabilisers and other excipients. It may additionally include other therapeutically active agents, in particular those of use in treating or preventing hepatitis C or related conditions.

The pharmaceutical composition may be in any suitable

form, depending on the intended method of administration. It may for example be in the form of a tablet, capsule or liquid for oral administration, or of a solution or suspension for administration parenterally.

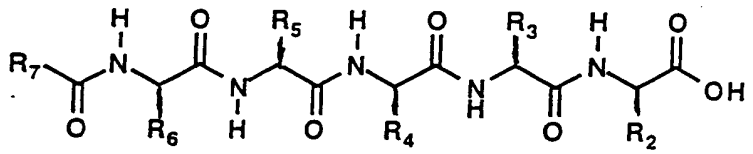
According to a fifth aspect of the invention, there is provided a method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, the method involving administering to a human or animal (preferably mammalian) subject suffering from the condition a therapeutically or prophylactically effective amount of a composition according to the fourth aspect of the invention, or of a compound or derivative according to the first aspect. "Effective amount" means an amount sufficient to cause a benefit to the subject or at least to cause a change in the subject's condition.

The dosage rate at which the compound, derivative or composition is administered will depend on the nature of the subject, the nature and severity of the condition, the administration method used, etc. Appropriate values can be selected by the trained medical practitioner. Preferred daily doses of the compounds are likely to be of the order of about 1 to 100 mg. The compound, derivative or composition may be administered alone or in combination with other treatments, either simultaneously or sequentially. It may be administered by any suitable route, including orally, intravenously, cutaneously, subcutaneously,etc. Intravenous administration is preferred. It may be administered directly to a suitable

site or in a manner in which it targets a particular site, such as a certain type of cell - suitable targeting methods are already known.

A sixth aspect of the invention provides a method of preparation of a pharmaceutical composition, involving admixing one or more compounds or derivatives according to the first aspect of the invention with one or more pharmaceutically acceptable adjuvants, and/or with one or more other therapeutically or prophylactically active agents.

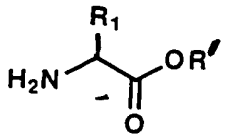
The compounds themselves may be prepared by reacting a compound of formula 4:



Formula 4

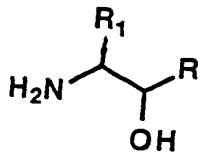
optionally in a protected form,

with an appropriate amine co-reactant (depending on the intended nature of R_1 and X in the final compound), examples of which include:



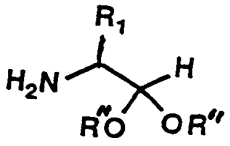
FORMULA 5

(for X = OH, as in compounds 1a, 1b and 1f), R' being a protecting group;



FORMULA 6

(for X = R or H, eg, as in compounds 1c, 1d, 1e, 1g, 1h 1j, 1k, 1L, or 1m); and



FORMULA 7

(for X = H, as in compound 1c, R" being a lower alkyl group such as methyl or ethyl).

Compounds of formula 1 having m=1 may be produced using homologs of the above compounds 5, 6 and 7 including an additional CH_2 group at the appropriate position.

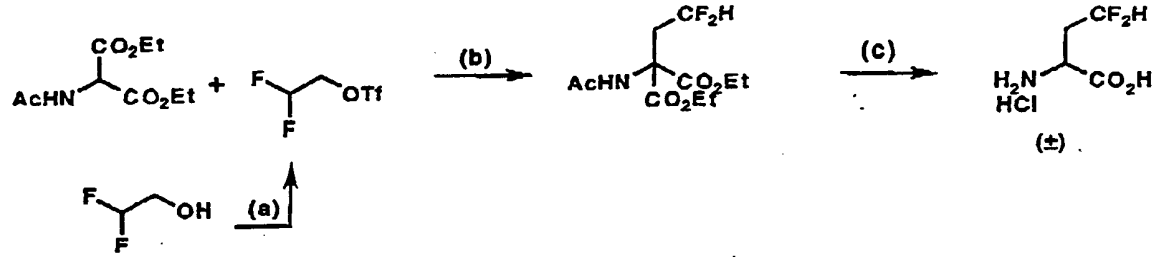
Compounds of formulae 5,6 and 7 may be used as racemates

or, alternatively, as individual D- or L-isomers. When a racemate is used subsequent separation of product diastereomers may be desirable.

In each case, the reaction can be carried out using standard methods of peptide synthesis. In the case of formula 6, oxidation of the alcohol to a ketone is also needed. In all cases, protecting groups may need to be removed, for instance under mildly acidic or basic conditions, to reach the final product.

A preferred compound of formula 5 is racemic 4,4-difluoro-2-aminobutyric acid. One possible scheme for the preparation of this compound is set out below in scheme 1

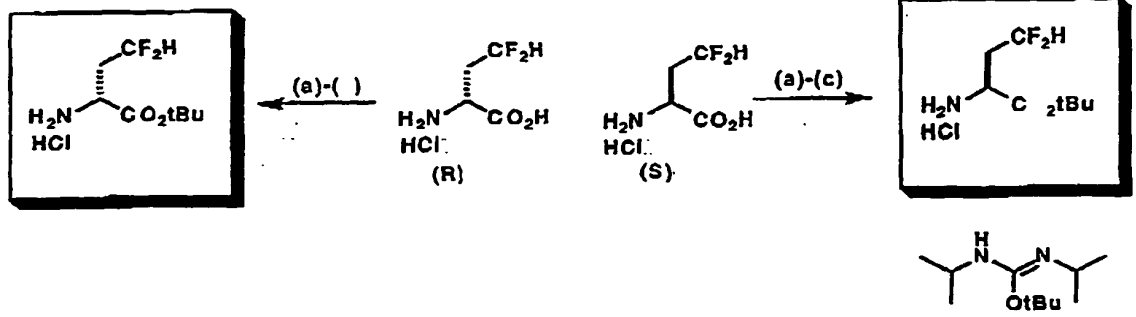
Scheme 1[a]



[a]Reagents: (a) Tf_2O, CH_2Cl_2, Et_3N; (b) KOtBu, THF, Δ; (c) 6N HCl, Δ

The individual R- and S- enantiomers of 4,4-difluoro-2-aminobutyric acid may be prepared from D- and L- aspartic acid, respectively using the method described by Winkler et al in Synthesis (1996), 1419-1421. The carboxylic acid group of these compounds may be protected, for instance by formation of t-butyl esters as shown below in scheme 2

Scheme 2[a]

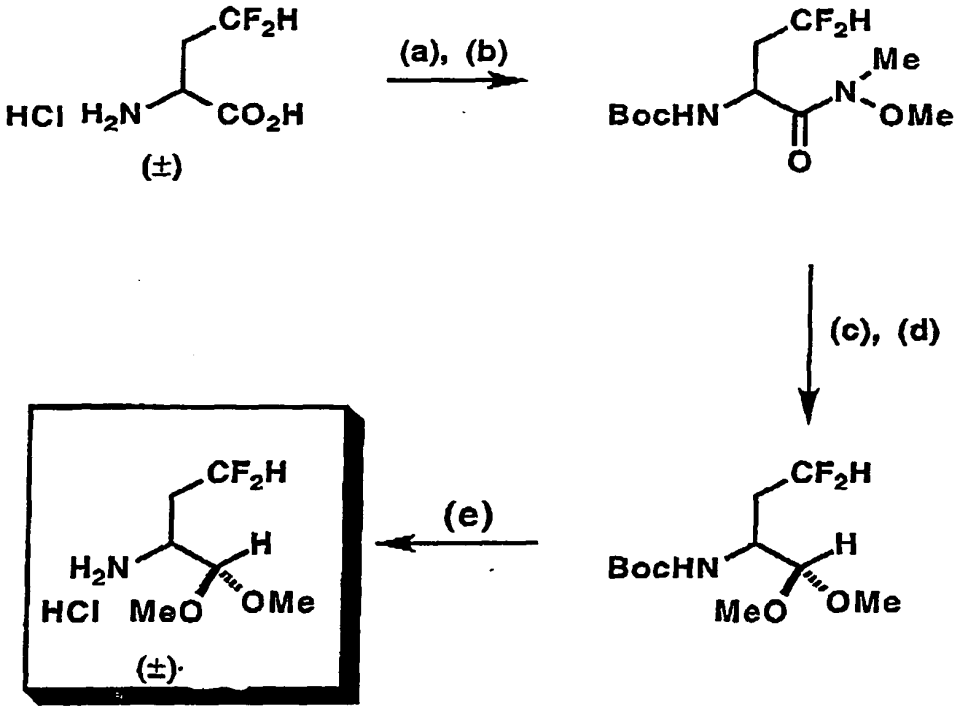


[a]Reagents: (a) CbzOSuc, Na_2CO_3, dioxan; (b) 10, CH_2Cl_2; (c) H_2, Pd/C, ether/HCl

10

One example of a racemic diacetal of formula 7 may be prepared as outlined below in scheme 3 which begins with racemic 4,4-difluoro-2-aminobutyric acid.

Scheme 3[a]

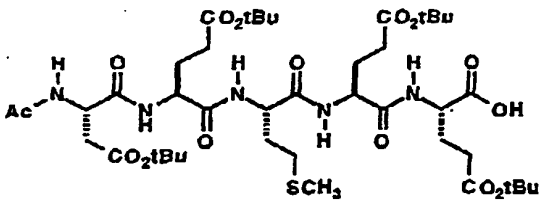


[a]Reagents: (a) Boc_2O; (b) NH(OMe)Me.HCl, EDCI.HCl, HOBt, iPr_2NEt; (c) DIBAL, THF, -78°; (d) $HC(OMe)_3$, TsOH; (e) HCl, M OH, 0°- rt

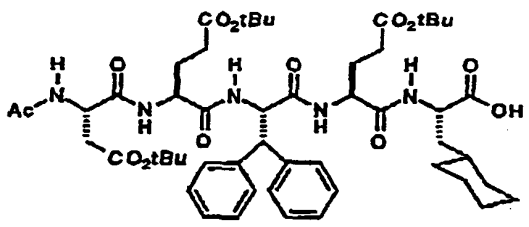
Compounds of formula 4 may be generated wholly or partly by chemical synthesis, and in particular can be prepared according to known peptide synthesis methods.

Preferably, all five amino acids, or amino acid analogs in the compound of formula 4 are L-isomers.

Preferably, the compound of formula 4 for reaction with a compound of formula 5,6 or 7 will be in protected form. For instance, any carboxylic acid groups other than that at the C terminus may preferably be protected, for instance as esters, eg as tertiary butyl esters. Examples of two highly preferred protected pentapeptides are set out below and labelled (A) and (B)



(A)



(B)

The invention provides, according to a seventh aspect, a method as described above for preparing a compound according to the first aspect.

An eighth aspect of the invention provides a compound of formula 4 (in which the definitions of R_2 - R_7 are as for compounds of formula 1), as an intermediate in a preparation method according to the seventh aspect.

Examples

Embodiments of the invention are described below by way of example only

(1) Synthesis

HPLC Conditions: Reversed phase analytical HPLC was performed on a Waters Symmetry C18 column (150 x 3.9 mm, 5 μm), flow rate 1 mL/min, using H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) as eluents; detection at 220 nm with a Waters 996 PDA detector. Gradient 1: linear, 90 A- 20% A 8 min, then in 2 min to 0% A, then isocratic. Gradient 2: linear, 70 - 40% A 10 min. . Gradient 3: linear, 90 - 70% A 10 min. Preparative HPLC was conducted on a Waters Symmetry C18 column (150 x 19 mm, 7μm) or a Waters Prep Nova-Pak HR C18 cartridge (40 x 100 mm, 6 μm) using H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) as eluents; detection at 220 nm with a Waters 486 absorbance detector.

EXAMPLE 1

Synthesis of compound 1a

i) 1,1-Difluoro-2-trifluoromethanesulfonyloxyethane

Triflic anhydride (120 g, 0.427 mol) was dissolved in anhydrous dichloromethane (70 mL) and cooled to -60° C. A solution of triethylamine (59.5 mL, 0.427 mol) and difluoroethanol (35 g, 0.427 mol) in dichloromethane (70

mL) was added slowly, so that the internal temperature did not exceed -50° C. After complete addition the resulting yellow solution was allowed to reach room temperature. Dichloromethane was distilled off under atmospheric pressure, and the remaining liquid fractioned under reduced pressure (70 - 80 mbar), using a 20 cm Vigreux column to give the title sulfonate (86.2 g, 94%) (b.p.: 58 - 60° C). ^{1}H-NMR ($CDCl_3$) 4.58 (dt, J = 3.6, 12.8 Hz, 2 H, CH_2), 6.05 (tt, J = 3.6, 54 Hz, 1 H, CHF_2); ^{19}F-NMR ($CDCl_3$) δ -74.6 (s), -127 (s).

ii) <u>Diethylacetamido-2-(2',2'-difluoroethyl) malonate</u>

Diethyl acetamido malonate (35.8 g, 0.165 mol) was dissolved in anhydrous THF (300 mL) and treated with potassium tert-butanolate (18.5 g, 0.165 mol) under vigorous stirring. The resulting suspension was refluxed for 1.5 h, and the above sulfonate (40 g, 0.187 mol) was added carefully *via* syringe to the refluxing suspension. The solution became homogeneous and was refluxed for another 3h. The solution was concentrated, and the residue dissolved in ethyl acetate and washed with hydrochloric acid (0.5 N, 2x), water (2x), saturated aqueous $NaHCO_3$, sodium hydroxide (1 N, 1x) and brine. Drying (Na_2SO_4) and evaporation left an orange oil, which was dissolved in diethyl ether (250 mL). The flask was kept at -20° C overnight. 32.6 g (70%) of a colourless solid was collected; mp 72 - 73 °C. ^{1}H-NMR ($CDCl_3$) δ 1.26 (t, J = 7.1 Hz, 6 H, CH_3), 2.05 (s, 3 H, $COCH_3$), 2.98 (dt, J = 4.7, 16.5 Hz, 2 H, CH_2CHF_2), 4.27 (q, J = 7.1 Hz, 4 H, CH_2), 5.85 (tt, J = 4.7, 55.8 Hz, 1 H, CHF_2), 6.90

(bs, 1 H, NH); ^{13}C-NMR ($CDCl_3$) δ 13.8, 22.9, 36.8 (t, *J* = 22.6 Hz), 62.8, 63.1, 115.2 (t, *J* = 239 Hz), 167.0, 169.7; ^{19}F-NMR ($CDCl_3$) δ -116.8 (s); MS *m/z* 282 (M^+ + H).

iii) (+)-2-Amino-4,4-difluorobutanoic acid hydrochloride

The malonate prepared above (32 g, 0.114 mol) was refluxed in 500 mL hydrochloric acid (6 N) overnight. The aqueous phase was extracted with diethyl ether and then evaporated to give the title compound (19.9 g; quantitative yield) as a colourless solid; mp 164 - 165 °C. ^{1}H-NMR (D_2O) δ 2.35 - 2.70 (m, 2 H, CH_2), 4.27 (dd, *J* = Hz, 1 H, CH), 6.19 (tt, *J* = Hz, 1 H, CHF_2); ^{13}C-NMR (D_2O) δ 34.0 (t, *J* = 22.2 Hz), 48.2, 115.7 (t, *J* = 238 Hz), 171.4; ^{19}F-NMR (D_2O) δ -112.7 (d, 287 Hz), -114.2 (d, 287 Hz); MS *m/z* 149 (M^+ + H).

iv) *(R)*-tert-Butyl-2-amino-4,4-difluoro butanoate hydrochloride

1.5 g (10.78 mmol) of (*R*) 2-Amino-4,4-difluoro butanoic acid (prepared as described in Winkler et al, Synthesis 1419 1996) was dissolved in aqueous half saturated Na_2CO_3 (50 mL) and cooled to 0 °C. A solution of (benzyloxycarbonyloxy)succinimide (2.69 g, 10.78 mmol) in dioxane (50 mL) was added dropwise over 30 min. The resulting suspension was stirred overnight at room temperature. After evaporation of the dioxane under reduced pressure water (20 mL) and EtOAc (150 mL) were added. The aqueous phase was brought to pH 2 by addition

of 1 N HCl, the organic phase was separated, washed with brine and dried. Evaporation gave 2.85 g (97%) of a colourless oil.

This material (950 mg; 3.55 mmol) was dissolved in dichloromethane (15 mL) and N,N'-isopropyl-O-tbutyl isourea (1.42 g, 7.10 mmol) was added dropwise. The solution was brought to gentle reflux. After 8 h another 1.42 g of the isourea was added and reflux was continued overnight. The diisopropylurea was removed by filtration, and the residue purified by flash chromatography (petroleum ether/ethyl acetate 10 : 1) to give a colourless oil (844 mg; 72%). ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR (DMSO-d_6) δ 1.38 (s, 9 H), 2.14 - 2.28 (m, 2 H), 4.08 (m, 1 H), 5.03 (d, J = 12.6 Hz, 1 H),), 5.06 (d, J = 12.6 Hz, 1 H), 6.10 (tt, J = 4.7, 56.2 Hz, 1 H), 7.27 - 7.39 (m, 5 H), 7.79 (d, J = 8.1 Hz, 1 H); ^{13}C-NMR (DMSO-d_6) δ 27.4, 34.9 (t, J = 22.5 Hz), 49.5, 65.5, 81.2, 115.9 (t, J = 238 Hz), 127.7, 127.8, 128.3, 136.7, 155.8, 169.8; ^{19}F-NMR (DMSO-d_6) δ -115.1 (d, J = 283 Hz), -115.8 (d, J = 283 Hz); MS m/z 330 (M^+ + H).

300 mg (0.91 mmol) of this material were hydrogenated over 10% palladium-on-charcoal in methanol (10 mL). After 5h, the catalyst was removed by filtration, then some ethyl acetate and a 1 N solution of hydrochloric acid in diethyl ether (1.37 mL) were added. After evaporation in vacuo the title compound (203 mg; 96%) was obtained as an off-white solid; mp 153 - 154 °C; ^{1}H-NMR (DMSO) δ 1.44 (s, 9 H), 2.38 - 2.50 (m, 2 H), 4.03 (t, J = 6.2 Hz, 1 H),

6.35 (tt, J = 4.3, 55.6 Hz, 1 H), 8.85 (bs, 3H); ^{13}C-NMR (DMSO-d_6) δ 27.3, 34.3 (t, J = 23.3 Hz), 47.6, 83.4, 114.9 (t, J = 238 Hz), 167.0; ^{19}F-NMR (DMSO-d_6) δ -114.5 (d, J = 285 Hz), -115.3 (d, J = 285 Hz); MS m/z 196 (M^+ + H).

v) (*S*)-tert.-Butyl-2-amino-4,4-difluoro-butanoate hydrochloride

Using the procedure described above for the (R)-enantiomer, the title compound was obtained as an off-white powder; mp 152 - 153 °C (MeOH, Et_2O, pentane); α_D +5.1° (c = 1.0, anhydrous MeOH). ^{1}H-NMR (DMSO-d_6) δ 1.44 (s, 9 H), 2.36 - 2.50 (m, 2 H), 4.05 (bs, 1 H), 6.31 (tt, J = 4.5, 55.6 Hz, 1 H), 8.71 (bs, 3H); ^{13}C-NMR (DMSO-d_6) δ 27.3, 34.3 (t, J = 23.3 Hz), 47.6, 83.5, 114.9 (t, J = 238 Hz), 167.1; ^{19}F-NMR (DMSO-d_6) δ -114.4 (d, J = 285 Hz), -115.2 (d, J = 285 Hz); MS m/z 196 (M^+ + H).

vi) (+)-(2-N-(tert-Butoxycarbonyl)-amino)-4,4-difluoro-butyric N-methyl-O-methylcarboxamide

1.0 g (5.7 mmol) of (±)-2-amino-4,4-difluoro butanoic acid hydrochloride was converted to its Boc derivative using di-tert.-butyl dicarbonate (1.24 g, 5.7 mmol). After extractive workup 1.16 g (85%) of a colourless solid was obtained, which was used without further purification; mp 127 - 129 °C. ^{1}H-NMR (DMSO-d_6) δ 1.37 (s, 9 H, t-Bu), 2.15 (m, 2 H, CH_2), 4.03 (m, 1 H, α-CH), 6.07

(tt, J = 4.5, 56 Hz, 1 H, CHF_2), 7.30 (d, J = 8.5 Hz, 1 H, NH), 12.80 (bs, 1 H,COOH); ^{13}C-NMR (DMSO-d_6) δ 28.0, 35.0 (t, J = 22 Hz), 48.4, 78.3, 116.0 (t, J = 238 Hz), 155.3, 172.5; ^{19}F-NMR (DMSO-d_6) δ -115.0 (d, J = 282 Hz), -115.7 (d, J = 282 Hz); MS m/z 240 (M^+ + H).

To a solution of the Boc derivative prepared above (1.59 g, 6.65 mmol), EDC•HCl (1.40 g, 7.32 mmol) and HOBt (1.08 g, 7.98 mmol) in anhydrous dichloromethane (30 mL) was added a solution of N,O-dimethylhydroxylamine hydrochloride (714 mg, 7.32 mmol) and diisopropylethylamine (1.74 mL, 9.98 mmol) in dichloromethane (20 mL) at 0 °C. After stirring at room temperature for 3 days, some dichloromethane was removed under reduced pressure. The resulting solution was diluted with ethyl acetate (150 mL) and washed successively with 1 N HCl (2x), sat. aqueous $NaHCO_3$ (2x) and brine. The organic extract was dried (Na_2SO_4) and concentrated in vacuo to give the title compound (1.81 g; 96%) of as a colourless solid. A small sample was recrystallized to give: mp 81 -82 °C. ^{1}H-NMR ($CDCl_3$) δ 1.44 (s, 9 H, t-Bu), 1.93 - 2.44 (m, 2 H, CH_2), 3.23 (s, 3 H, NCH_3), 3.76 (s, 3 H, OCH_3), 4.84 (m, 1 H, α-CH), 5.39 (bd, J = 9.0 Hz, 1 H, NH), 5.95 (ddt, J = 3.6, 5.8, 56.0 Hz, 1 H, CHF_2); ^{13}C-NMR ($CDCl_3$) δ 28.3, 32.3, 37.6 (t, J = 22 Hz), 46.3, 61.7, 80.2, 115.3 (t, J = 239 Hz), 155.3, 171.2; ^{19}F-NMR ($CDCl_3$) δ -114.6 (d, J = 287 Hz), -115.5 (d, J = 287 Hz); MS m/z 283 (M^+ + H).

(vii) <u>(+)-2-(N-tert.-Butoxycarbonyl)amino-4,4-</u>

difluorobutyraldehyde dimethylacetal

To a solution of the above urethane (4.89 g, 17.32 mmol) in tetrahydrofuran (100 mL) was added neat diisobutylaluminum hydride (6.79 mL, 38.11 mmol) dropwise at -78 °C. The solution was stirred for 2.5 h at this temperature, then methanol (5 mL) was added dropwise and the cooling bath removed. The solution was diluted with ethyl acetate (500 mL) and then washed successively with ice-cold 1 N HCl (150 mL, 3x), 2 N aqueous Rochelle's salt (150 mL) and brine (2x). Drying of the organic extract (Na_2SO_4) and evaporation in vacuo gave 3.47 g (90%) of (±)-2-(N-tert.-Butoxy carbonyl)-amino-4,4-difluoro butyraldehyde as an opaque oil, which was used in the next step without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.47 (s, 9 H, t-Bu), 2.25 (m, 1 H, CH_2), 2.55 (m, 1 H, CH_2), 4.31 (m, 1 H, α-CH), 5.33 (bs, 1 H, NH), 6.03 (dt, J = 6.0, 56 Hz, 1 H, CHF_2), 9.60 (s, 1 H, CHO);

1.8 g (8.06 mmol) of the crude aldehyde were converted into the dimethylacetal using trimethylorthoformate (12.4 mL, 112.9 mmol) and p-toluenesulfonic acid (154 mg, 0.81 mmol) in anhydrous methanol (30 mL). After stirring overnight at room temperature, TLC (petroleuim/ethyl acetate 2:1) indicated complete consumption of the aldehyde. Saturated aqueous $NaHCO_3$ was added and the methanol evaporated under reduced pressure. The residue was dissolved with ethyl acetate (200 mL) and washed successively with saturated aqueous $NaHCO_3$ and brine. Drying (Na_2SO_4) and evaporation left an oil which was

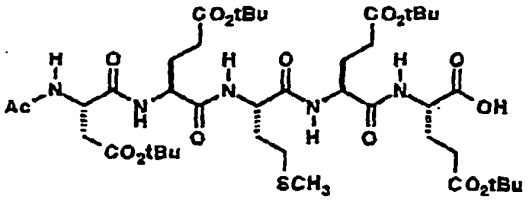
purified by flash chromatography (160 g silica gel, petroleum/ethyl acetate 4 : 1, containing 0.5% triethylamine), to give the title compound (1.44 g; 66%) as a colourless solid; mp 61 -62 °C. ^{1}H-NMR ($CDCl_3$) δ 1.48 (s, 9 H, t-Bu), 1.86 - 2.05 (m, 1 H, CH_2), 2.09 - 2.27 (m, 1 H, CH_2), 3.44 (s, 3 H, OCH_3), 3.45 (s, 3 H, OCH_3), 3.99 (m, 1 H, α-CH), 4.25 (d, J = 3.0 Hz, 1 H, $CH(OMe)_2$), 4.76 (bd, J = 8.0 Hz, 1 H, NH), 5.96 (ddt, J = 4.0, 5.4, 56.6 Hz, 1 H, CHF_2); ^{13}C-NMR ($CDCl_3$) δ 28.3, 34.4 (t, J = 22 Hz, CH_2CHF_2), 47.6, 55.9, 56.5, 79.8, 105.6, 116.3 (t, J = 238 Hz, CH_2CHF_2), 155.5; ^{19}F-NMR ($CDCl_3$) δ -114.6 (d, J = 284 Hz), -115.5 (d, J = 284 Hz); MS m/z 270 (M^+ + H).

viii) <u>(+)-2-Amino-4,4-difluorobutyraldehyde dimethylacetal hydrochloride</u>

To 300 mg (1.11 mmol) of the above acetal was added a solution of gaseous HCl in anhydrous methanol (10% HCl by weight) at 0 °C. The solution was stirred at this temperature for 30 min, then 20 min at ambient temperature. Evaporation and drying under high vacuum gave the title compound (200 mg) as a yellow oil.

(ix) <u>Ac-Asp-Glu-Met-Glu-Glu-Difluoroaminobutyric acid (1a)</u>

The protected pentapeptide shown below (ac-tert-butyl-asp-tert-butyl-glu-met-tert-butyl-glu-tert-butyl-glu) was employed in this example



(A)

30 mg pentapetide (0.03 mmol) was dissolved in dichloromethane (0.5 mL) and cooled to 0 °C. N-Ethyl, N'-(dimethylamino)propylcarbodiimide hydrochloride (EDCI.HCl) (6.3 mg, 0.033 mmol) and hydroxybenzotriazole (HOBT) (4.9 mg, 0.036 mmol) were added, followed by solid (s)-tert-butyl-2-amino-4,4-difluoro-butanoate hydrochloride (from v, above) (10.4 mg, 0.045 mmol) and diisopropylethylamine (11 µL, 0.06 mmol). The resulting solution was stirred overnight at room temperature, then taken into ethyl acetate (50 mL) and washed successively with 1 N HCl (2x 25 mL), saturated aqueous $NaHCO_3$ (2 x 20 mL), and brine. Drying (Na_2SO_4) and evaporation gave a solid which was immediately treated with a solution of trifluoroacetic acid, dichloromethane and water (60/30/10, v/v/v; 10 mL). After 30 min at room temperature the solvents were evaporated in vacuo and the remaining solid separated by preparative HPLC (Waters Symmetry column). Flow 17 mL/min; Gradient : linear, 90% A, 3 min isocratic, in 15 min to 75% A; 7 mg of crude per injection. The product, compound 1a (RT 10.4 min); 12 mg (50%) was obtained as a colourless solid after lyophilization.

^{1}H-NMR (DMSO-d_6) δ 1.73 - 1.95 (m, 8 H), 1.83 (s, 3 H), 2.02 (s, 3 H), 2.19 - 2.30 (m, 8 H), 2.35 -2.48 (m, 3 H), 2.61 (dd, J = 5.2, 11.7 Hz, 1 H), 4.14 - 4.26 (m, 3 H),

4.29 (m, 1 H), 4.36 (m, 1 H), 4.50 (dd, J = 5.4, 7.7 Hz, 1 H), 6.05 (ddt, J = 4.6, 51.6 Hz, 1 H), 7.92 (d, 1 H, J = 8.4 Hz, 1 H), 7.96 (d, 1 H, J = 8.2 Hz, 1 H), 7.99 (m, 2 H),), 8.18 (d, 1 H, J = 7.5 Hz, 1 H), 8.33 (bd, 1 H, J = 7.0 Hz, 1 H), 11.9 - 12.4 (bs, 5 H); ^{19}F-NMR (DMSO-d_6) δ -115.0 (d, J = 282 Hz), -115.8 (d, J = 284 Hz); MS m/z 815 (M^+ + H).

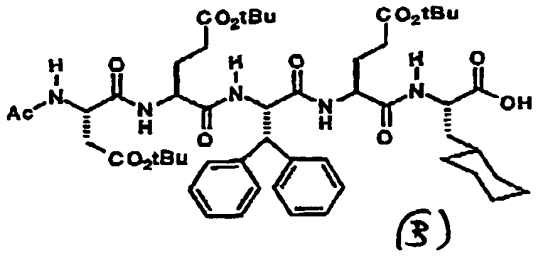
EXAMPLE 2

Synthesis of compound 1b[1]

Ac-Asp-Glu-Diphenylalanine-Glu-β-Cyclohexylala-Difluoroaminobutyric acid (1b[1])

In this example, (S)-tert-butyl-2-amino-4,4-difluoro-butanoate was used in the preparation of a first diastereomer of compound 1b.

This example, and also examples 3 and 4 below, employed the protected pentapeptide shown below (ac-tert-butyl-asp-tert-butyl-glu-diphenylala-tert-butyl glu-β-cyclohexyl-ala)



(S)

50 mg pentapetide (0.05 mmol) was dissolved in DMF (0.5 mL) and cooled to 0 °C. HATU and solid (S)-tert-butyl-2-amino-4,4-difluoro-butanoate hydrochloride (from v, above) were added, followed by 2,6-lutidine (24 µL, 0.2 mmol). The reaction was allowed to reach room temperature and stirred for 3 h. Analytical HPLC (gradient 1) indicated incomplete conversion of the pentapeptide (~30% remaining, RT 10.4 min, gradient 1, product 11.9 min). After another 2 h the mixture was taken into ethyl acetate (100 mL) and washed successively with 1 N HCl, (2x 50 mL), saturated aqueous $NaHCO_3$ (2 x 50 mL), and brine. Drying (Na_2SO_4) and evaporation gave a light yellow solid which was immediately deprotected with a solution of trifluoroacetic acid, dichloromethane and water (60/30/10, v/v/v; 10 mL). After 30 min at room temperature the solvents were evaporated in vacuo and the remaining solid separated by preparative HPLC (Waters Symmetry column). Flow 17 mL/min; Gradient : linear, 68% A, 3 min isocratic, in 17 min to 65% A; 6 mg of crude per injection. The first peak was deprotected pentapetide (RT 11.6 min), the second the desired product compound 1b (RT 12.2 min); 11 mg (23%) of a colourless solid after lyophilization.

^{1}H-NMR (DMSO-d_6) δ 0.76-0.95 (m, 2 H), 1.08 - 1.32 (m, 4 H), 1.32 - 1.41 (m, 1 H), 1.42 - 1.51 (m, 1 H), 1.53-1.80 (m, 9 H), 1.83 (s, 3 H), 1.97 - 2.35 (m, 6 H), 2.38 - 2.50 (m, 2 H), 4.04 - 4.13 (m, 2 H), 4.13 - 4.21 (m, 1 H), 4.27 - 4.37 (m, 1 H), 4.38 (d, J = 10.3 Hz, 1 H), 4.47 (m, 1 H), 5.19 (app. t, J = 9.5 Hz, 1 H), 6.04 (ddt, J = 4.0, 5.7, 56.2 Hz, 1 H), 7.05-7.33 (m, 10 H), 7.75

(d, 1 H, J = 7.3 Hz, 1 H), 7.79 (d, 1 H, J = 8.0 Hz, 1 H), 7.89 (d, 1 H, J = 8.1 Hz, 1 H),), 7.96 (d, 1 H, J = 7.6 Hz, 1 H), 8.10 (d, 1 H, J = 7.0 Hz, 1 H), 8.10 -8.12 (bs, 1 H); MS m/z 929 (M^+ - H).

EXAMPLE 3

Synthesis of compound 1b²

In this example, (R)-tert-butyl-2-amino-4,4-difluorobutanoate hydrochloride (from iv, above) was used to prepare the alternative diastereomer to that prepared in example 2. The method was as described in example 2.

After 3 h analytical HPLC indicated only minor amounts of the protected pentapeptide. After workup the crude product was deprotected as described in example 2 and separated by preparative HPLC (Waters Symmetry column). Flow 17 mL/min; Gradient : linear, 70% A, 3 min isocratic, in 12 min to 40% A; 6 mg of crude per injection. 22 mg (47%) of 17 (RT 9.2 min) as a colourless solid were obtained after lyophilization.

^{1}H-NMR (DMSO-d_6) δ 0.77-0.91 (m, 2 H), 1.06 - 1.25 (m, 4 H), 1.29 - 1.36 (m, 1 H), 1.37 - 1.44 (m, 1 H), 1.52-1.80 (m, 9 H), 1.82 (s, 3 H), 1.99 - 2.13 (m, 4 H), 2.16 - 2.33 (m, 2 H), 2.42 (dd, J = 8.8, 16.6 Hz, 1 H, β-CH_2 Asp), 2.49 (under DMSO, m, 1 H), 4.08 (m, 2 H), 4.21 (m, 1 H), 4.33 (m, 1 H), 4.37 (d, J = 10.3 Hz, 1 H), 4.47 (m, 1 H), 5.21 (app. t, J = 9.4 Hz, 1 H), 5.99 (dt, J = 4.6,

56.3 Hz, 1 H), 7.05-7.40 (m, 10 H), 7.65 (d, 1 H, J = 7.7 Hz, 1 H), 7.78 (d, 1 H, J = 7.9 Hz, 1 H), 7.87 (d, 1 H, J = 8.4 Hz, 1 H),), 7.96 (d, 1 H, J = 7.8 Hz, 1 H), 8.14 (d, 1 H, J = 7.7 Hz, 1 H), 8.30 (d, 1 H, J = 8.10 Hz, 1 H), 11.90 - 12.30 (bs, 4 H); MS m/z 929 (M^+ - H).

EXAMPLE 4

Synthesis of compound 1c

220 mg of the protected pentapeptide (Ac-tert-butyl-asp-tert-butyl-glu-diphenylalanine-tert-butyl-glu-β-cyclohexylala) (0.225 mmol) were dissolved in 1 mL chloroform. EDC•HCl (52 mg, 0.27 mmol) and HOBt (61 mg, 0.45 mmol) were added and the solution cooled to 0 °C. The acetal, (± 2-amino-4,4-difluorobutyraldehyde dimethylacetal hydrochloride (from viii above) (80 mg, 0.39 mmol) was dissolved in chloroform (0.8 mL) containing Hünig's base (0.47 mmol, 0.082 mL) and the resulting solution was added via syringe to the pentapeptide. Another 0.3 mL chloroform was used to rinse flask and syringe. The cooling bath was removed after 10 min and the orange solution stirred for 3 h. Analytical HPLC indicated complete conversion of the pentapetide. The reaction was taken into a mixture of ethyl acetate and dichloromethane (150 mL, 3 : 1) and washed successively with 0.1 M aqueous $KHSO_4$, (3x 80 mL), water (2x 100 mL), saturated aqueous $NaHCO_3$ and brine (2x 100 mL). Drying (Na_2SO_4) and evaporation gave a brown solid which was immediately deprotected with a solution of trifluoroacetic acid, dichloromethane and water (60/35/5,

v/v/v; 50 mL). After 30 min at room temperature the solvents were evaporated in vacuo and the remaining brown solid (252 mg) comprising a mixture of diastereomers was separated by preparative HPLC (Nova-Pak Prep column). Flow 40 mL/min; Gradient : linear, 70% A, 2 min isocratic, in 18 min to 60% A; 20 mg of crude per injection.

First fraction: RT: 9.4 min, 54 mg (26%) of a colourless powder after lyophilization; 1 diastereomer, 94% pure by analytical HPLC (gradient 1, 6.77 min; gradient 2, 6.45 min). In the ^{1}H-NMR 10 - 20% of the aldehyde was visible as its hydrate. Addition of water gave a ratio of aldehyde to hydrate of 1 : 9. Only data for the aldehyde are reported. ^{1}H-NMR (DMSO-d_6) δ 0.77 - 0.94 (m, 2 H), 1.05 - 1.31 (m, 4 H), 1.32 - 1.50 (m, 2 H), 1.52 - 1.78 (m, 9 H), 1.82 (s, 3 H), 1.95 - 2.15 (m, 6 H), 2.36 - 2.46 (m, 2 H, β-CH_2 Asp), 4.00 - 4.06 (m, 2 H, α-CH difluoro, Glu),), 4.12 - 4.23 (m, 2 H, α-CH Cha, Glu), 4.39 (d, J = 10.3 Hz,_1 H, β-CH Dif), 4.47 (m, 1 H, α-CH Asp), 5.19 (app. t, J = 9.4 Hz, 1 H, α-CH Dif), 6.10 (dt, J = 4.6, 56.0 Hz, 1 H, CHF_2), 7.05 - 7.38 (m, 10 H), 7.75 (d, J = 7.3 Hz, 1 H, NH difluoro), 7.81 (d, J = 6.9 Hz, 1 H, NH Cha), 7.86 (d, J = 8.0 Hz, 1 H, NH Dif), 8.10 (m, 2 H, NH Glu), 8.40 (d, J = 7.2 Hz, 1 H, NH Asp), 9.26 (s, 1 H, CHO), 11.50 - 12.50 (bs, 3 H, COOH); MS m/z 915 (M^+ + H). HRMS (Fab) ($C_{44}H_{56}F_2N_6O_{13}$) calc. 914.3873, fd.

Second fraction: RT: 12.2 min, 42 mg (20%), colourless powder after lyophilization;

^{1}H-NMR (DMSO-d_6) δ 0.76 - 0.94 (m, 2 H), 1.05 - 1.30 (m, 4 H), 1.32 - 1.50 (m, 2 H), 1.52 - 1.78 (m, 9 H), 1.83 (s, 3 H), 1.95 - 2.15 (m, 6 H), 2.25 - 2.45 (m, 2 H), 3.98 - 4.12 (m, 2 H),), 4.15 - 4.23 (m, 2 H), 4.35 - 4.51 (m, 2 H), 5.15 - 5.19 (m, 1 H), 6.06 (dt, *J* = 4.5, 56.1 Hz, 1 H), 7.07 - 7.38 (m, 10 H), 7.58 (d, *J* = 7.5 Hz, 1 H), 7.60 - 8.12 (m, 4 H), 8.43 (bs, 1 H), 9.32 (s, 1 H, CHO), 11.90 (bs, 3 H, COOH); MS *m/z* 915 (M^+ + H).

Using analogous methods, compounds 1d to 1m were also produced.

2. INHIBITION OF NS3 PROTEASE

The ability of the compounds to inhibit NS3 protease was evaluated using an NS3/4A complex comprising the NS3 protease domain and a modified form of the NS4A peptide, Pep 4AK [KKKGSVVIVGRIILSGR(NH_2)]. As substrate, a substrate peptide 4AB [DEMEECASHLPYK] based on the sequence of the NS4A/NS4B cleavage site of the HCV polyprotein, was used

Cleavage, assays were performed in 57μl 50 mM Hepes pH7.5, 1 % CHAPS, 15 % glycerol, 10 mM DTT (buffer A), to which 3μl substrate peptide were added. As protease co-factor a peptide spanning the central hydrophobic core (residues 21-34) of the NS4A protein, Pep4AK [KKKGSVVIVGRIILSGR(NH_2)] was used. Buffer solutions containing 80 μM Pep4AK were preincubated for 10 minutes with 10-200 nM protease and reactions were started by

addition of substrate. Six duplicate data points at different substrate concentrations were used to calculate kinetic parameters. Incubation times were chosen in order to obtain <7% substrate conversion and reactions were stopped by addition of 40 µl 1 % TFA. Cleavage of peptide substrates was determined by HPLC using a Merck-Hitachi chromatograph equipped with an autosampler. 80 µl samples were injected on a Lichrospher C18 reversed phase cartridge column (4 x 74mm, 5µm, Merck) and fragments were separated using a 10-40 % acetonitrile gradient a 5%/min using a flow rate of 2.5ml/min. Peak detection was accomplished by monitoring both the absorbance at 220nm and tyrosine fluorescence (λ_{ex} = 260 nm, λ_{em} = 305nm). Cleavage products were quantitated by integration of chromatograms with respect to appropriate standards. Kinetic parameters were calculated from nonlinear least-squares fit of initial rates as a function of substrate concentration with the help of a Kaleidagraph software, assuming Michaelis-Menten kinetics.

K_1 values of peptide inhibitors were calculated from substrate titration experiments performed in the presence of increasing amounts of inhibitor. Experimental data sets were simultaneously fitted to eq.1 using a multicurve fit macro with the help of a Sigmaplot software:

$$V = (V_{max}S)/(K_m(1+K_i/I)+S); \qquad \text{(eq.1)}$$

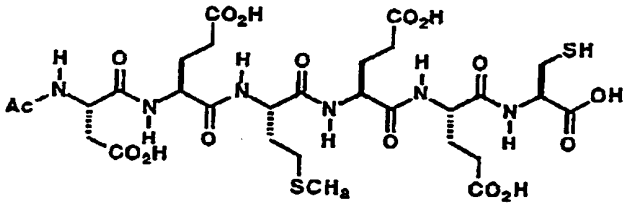
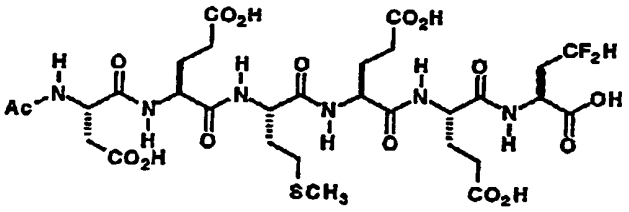
Alternatively, K_i values were derived from IC50 values, calculated using a four-parameter logistic function,

according to eq.2:

$$IC50 - (1+S/K_m)K_i \qquad \text{(eq.2)}$$

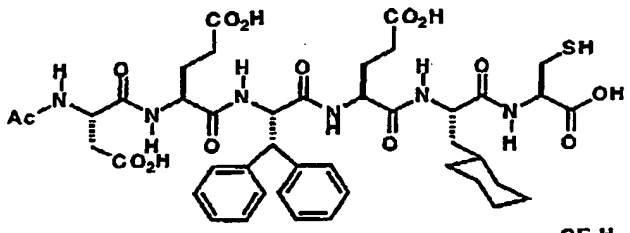
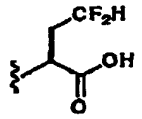
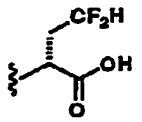
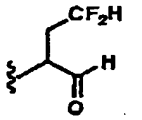
Results for the compounds synthesized in Examples 1 to 4 above are tabulated below in Tables 1 and 2. In Table 2, the data shown for compound 1c are for the more active diastereomer.

Table 1. Inhibition of NS3 Protease[a]

cmpd #	K_i
	0.7
 1a	1.5

[a] K_i values (μM) are given for NS3 protease inhibition.

Table 2. Inhibition of NS3 Protease

	cmpd #	K_i
		40
	1b¹	21
	1b²	640
	1c	0.5

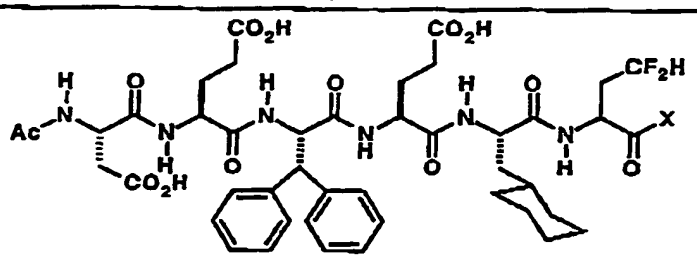
K_i values (nM) are given for NS3 protease inhibition

As shown in the tables, the difluoro analogs 1a (K_i 1.5μM) and 1b¹ (K_i 21nM) had essentially equal affinity to their cysteine counterparts (Ki 0.7μM and 40nM respectively). 1b², the P_1 diastereomer of 1b¹ had reduced affinity.

The aldehyde 1c had K_i 0.5nM, and was a reversible competitive inhibitor with a slow K_{off} (1.4×10^{-3} S^{-1}) typical of covalent inhibitors of serine protease. Table 3 shows a comparison of the kinetic parameters of the acid 1b¹ with the aldehyde analogue 1c. The relatively long half-life of the protease-aldehyde complex (8 mins) compared to the (presumably) non-covalent protease-acid complex (<10s) is consistent with the formation of a

covalent bond between the active site serine and the aldehyde carbonyl group.

Table 3. Comparison of kinetic parameters for a non-covalent (1b) and covalent (1c) inhibitors of NS3 protease[a]



cmpd	X	K_i (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	$t_{1/2}$
1b	OH	21	2.2×10^6	nd	< 10 s
1c	H	0.5	2.6×10^6	1.4×10^{-3}	8 min

[a]K_i values, k_{on} (on-rate), K_{off} (off-rate), and $t_{1/2}$ (half-life of complex) are given for NS3 protease inhibition.

IC_{50} values were determined for all of compounds 1a to 1m and are set out overleaf in Table 4.

TABLE 4

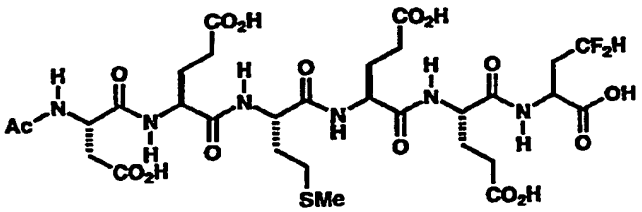
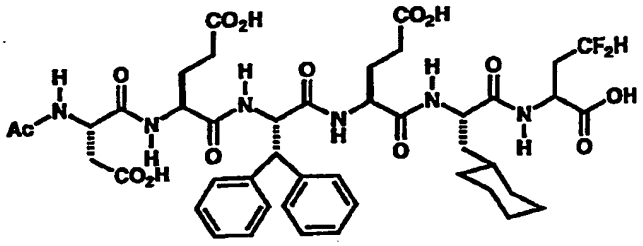
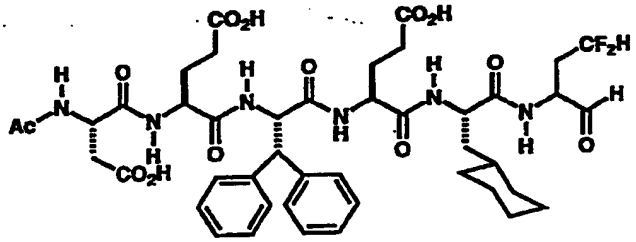
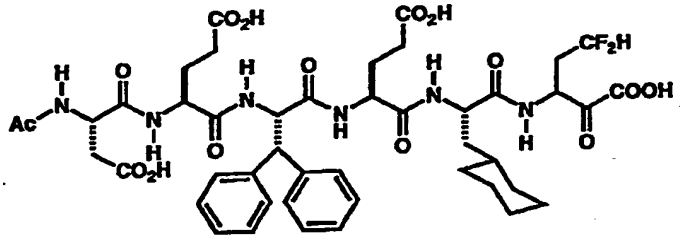
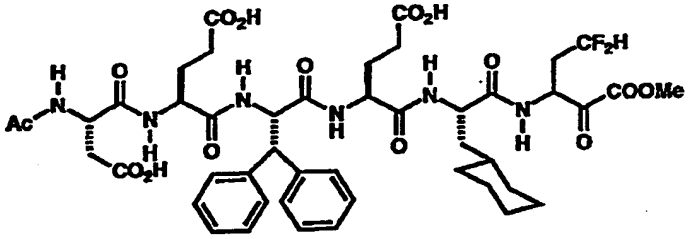
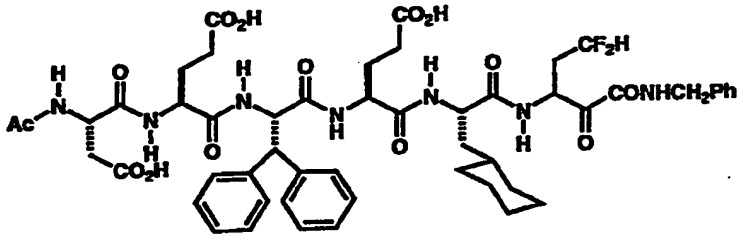
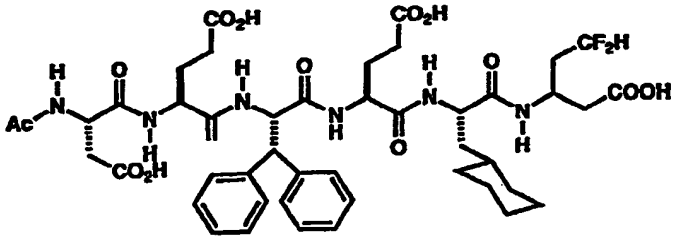
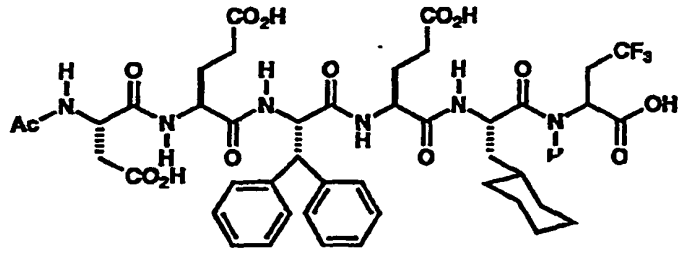
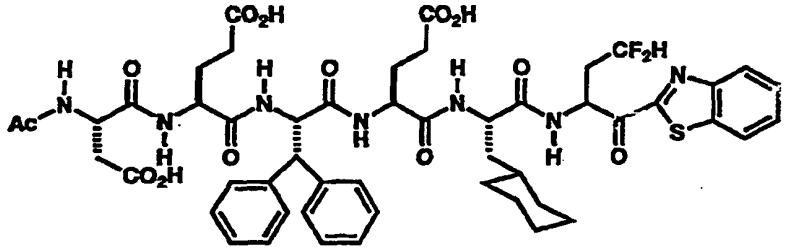
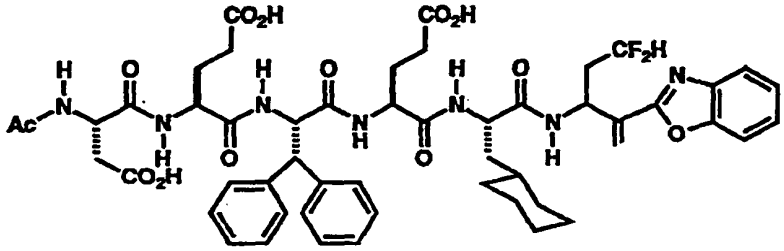
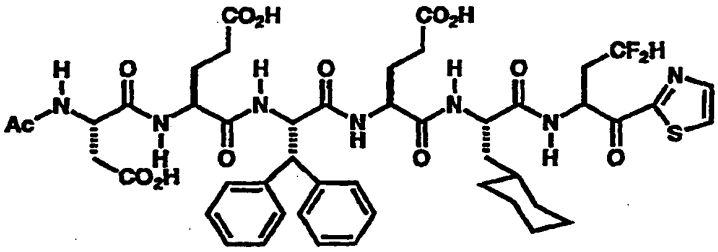
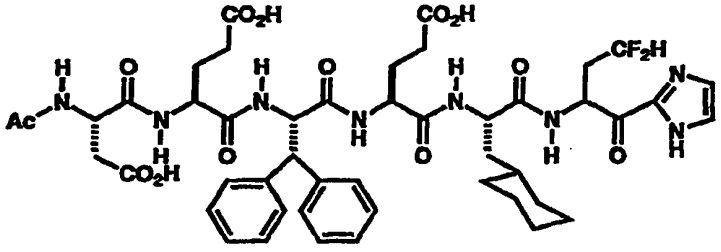
Compound	IC_{50}
	1a	3 μM
	1b1 (L) 1b2 (D)	20 nM 1 μM
	1c (L) (D)	0.5 nM 43 nM
	1d	0.4 nM
	1j	800 nM
	1e	9 nM
	1k	3 μM

TABLE 4 continued

	1f	110 nM
	1g	125 nM
	1i	600 nM
	1h	1 μM
	1m	6 μM

Notes to table:

Compounds 1d, 1j, 1e and 1k were produced as diasteremoeric mixtures which were subsequently separated

by chromatography. The IC_{50} value given is for the more active diastereomer which is presumed to have L stereochemistry at the P1 position.

The data for compound 1f are for the diastereomer having L stereochemistry at position 1.

Data for compounds 1g, 1l, 1h and 1m are for diastereomeric mixtures.

PCT/GB 99/01211

Newsum f in

9.6.99.